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Review

Engrailed homeobox transcription factors as potential markers and targets in cancer

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ABSTRACT

***Engrailed* (*En*) is a member of the homeobox gene family, which encodes a homeodomain-containing transcription factor that is essential during early development. The only known site of normal adult Engrailed protein (EN) expression is in the nervous system, and it has been implicated in the development of both young-onset Parkinson's disease as well as autism. Over-expression of EN has been linked to tumour development in adults, particularly in breast, prostate, melanoma and ovarian cancers, and there is a growing interest in its role as a diagnostic and prognostic biomarker. It is hoped that further work may confirm associations between *En* expression and therapy-resistant, poor prognosis cancers, similar to that identified with other homeobox gene profiles.**

1. Introduction

The homeobox genes are a superfamily of regulatory genes that encode homeodomain-containing transcription factors. They play a key role in early embryonic development but have also been linked to disease, including cancer. This review will aim to give an overview of homeobox gene function, particularly focusing on the biology of *Engrailed* (*En*), its role in disease including malignancy, and its potential as a biomarker.

The homeobox refers to a 183-bp DNA sequence, originally identified in *Drosophila*, which encodes a 61-amino-acid homeodomain within HOX proteins, enabling recognition and binding of sequence-specific DNA motifs. The specificity of this binding allows the protein to activate or repress the expression of many downstream effector target genes [1].

Over 100 homeobox genes have been identified, all of which share sequence similarity within the homeodomains, and have been separated into individual groups such as the *HOX* gene, the paired (*PAX*) gene, and the *Engrailed* (*En*) gene families. It is currently thought that homeobox genes represent 0.1–0.2% of the entire vertebrate genome. The role of homeobox-containing genes has been reported in vertebrate embryonic development. Specifically, they have been shown to be involved in the control of cell identity, cell growth and differentiation, as well as cell–cell and cell–extracellular matrix interactions. In mammals this extends to central nervous system, skeletal and limb development, as well as organogenesis. Many genetic disorders have been associated with deregulation of the homeobox genes, affecting multiple organ systems, and this can involve an up- or down-regulation of expression [2]. Analysis of the homeobox gene abnormalities has demonstrated the presence of deletion, insertion and substitution mutations.

Thirty-nine *HOX* genes have been identified in humans and are implicated in apoptosis, receptor signalling, differentiation, motility and angiogenesis [2]. They are fundamental to normal limb and organ development along the anterior–posterior axis [3], as well as blood vessel formation and prostate gland development [4]. In the normal adult human, specific patterns of *HOX* gene expression are involved in stem cell function as well as haematopoietic lineage differentiation, particularly the *HOXA10*, *HOXB3* and *HOXC4* genes [5]. In addition, expression of *HOXA10* and *HOXA11* varies during the course of the human menstrual cycle, with the most drastic increase occurring at the time of implantation. This continual process mirrors the HOX gene expression seen in the developing reproductive tract [6].

Abbreviations: En, Engrailed gene; En, Engrailed protein

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Mutations in human HOX genes have been linked with developmental limb malformations such as synpolydactyly (*HOXD13*) [7], radio-ulnar synostosis (*HOXA11*) [8] and the hand-foot-genital syndrome (*HOXA13*) [9], as well as malignancy. The *HOXA9* gene is associated with a form of acute myeloid leukaemia [10], and certain lymphoblastic leukaemias, and confers a worse prognosis and treatment failure [11]. *HOXC4–6* are expressed in non-Hodgkin's lymphomas [12]. The *HOX* gene family have also been implicated in the development of kidney tumours (*HOXA9, -C9, -D10*) [13], colon (*HOXA9, -B6–8, -C8–9, -D11*) [14], lung (*HOXA1,-A7, -A9, -C5*) [15], breast (*HOXA5, -A10, -B7, -B13*) [16], prostate (*HOXB13, -C8*) [17,18], melanoma (*HOXB7*) [19,20], endometrial (HOXD10) [21] and ovarian cancers (*HOXA7, -A9–11, -B13*) [22,23]. Certain gene expression profiles are associated with therapy-resistant cancers, and an increased risk of distant metastasis at recurrence [24].

Genetic control of the vertebrate anterior head and rostral brain development is influenced by *Emx* and *Otx* genes, with heterozygous mutations in the human *EMX2* gene contributing to schizencephaly, a rare congenital brain malformation accompanied by craniofacial defects [25]. The *MSX* gene family are closely linked with craniofacial and tooth development and MSX2 has been implicated in melanoma cell invasion and survival, with cytoplasmic expression indicating improved prognosis [26].

PAX genes are mostly involved in the control of embryonic tissue development and cellular differentiation, including renal morphogenesis (*PAX2, -8*), B-lymphocyte differentiation (*PAX5*), and central nervous system development. They show a strong correlation with human diseases, in particular renal defects and Wilms' tumours (*PAX2, -8*), microcephaly (PAX6), glioblastoma multiforme (*PAX5*), rhabdomyosarcoma (*PAX3*), congenital cataracts and other eye defects (*PAX6*), prostate cancer (*PAX2*) and thyroid cancers (*PAX8*) [27].

2. The biology of Engrailed

Engrailed (*En*) is another member of the homeobox gene family, which was first characterised in *Drosophila*. The encoded homeodomain-containing transcription factor again plays an important role in development, and has been identified in annelids [28], molluscs [29], insects [30], echinoderms, chordates [31], and vertebrates [32]. Although these genes share a limited degree of sequence conservation at the protein level, there are five *En* homology regions (EHs) which represent particular regions of similarity [33] (Fig. 1). Tolkunova et al. demonstrated that EH1 mediates transcriptional repression by recruiting the co-repressor *groucho*, and EH5 has a similar role [34]. EH4 is the homeodomain, which demonstrates the highest level of conservation [33], whilst EH2 and EH3 bind PBX, another homeodomain-containing transcription factor. The latter is able to modify the DNA binding affinity and specificity of *En* [35,36]. In addition to transcription, En protein may have a regulatory role in translation, as it is able to bind directly to the eukaryotic translation initiation factor 4E (eIF4E) with high affinity and specificity [37].

The homeodomain sequences also facilitate the association of cytoplasmic En protein with vesicles, enabling secretion of the protein from the cell [38–40]. En can also undergo internalization by the cell, a mechanism dependent upon sequences within the homeodomain [41]. This process has been demonstrated both at 4 and 37 °C and does not appear to require a specific receptor, although both the secretion and internalization mechanisms have yet to be fully elucidated [42,43].

Following its identification in *Drosophila*, where the mutated *En* resulted in a malformation of the border between the posterior and anterior wing compartments [44], further evaluation of its role in embryological neural and axonal development has taken place. In vertebrates, two *En* genes were discovered, *En1* and *En2*, which differ slightly in their specific functions [45,46]. High levels of both *En1* and *En2* are expressed in the alar (dorsal) cells of the midbrain/hindbrain border region during brain development, and influence the survival of mesencephalic dopaminergic neurons [47–50]. Wilson et al. recently demonstrated that *En1* and *En2* are expressed in many cell types in the cerebellum, with expression still evident at postnatal day 21 [51]. Between this point and the embryonic stages, there is a distinct change in their cellular and spatial distributions, with the expression domains becoming distinct. *En* plays a pivotal role in axonal guidance, as demonstrated in the developing chick optic tectum [52], where *En2* helps to establish rostro-caudal polarity. The EN2 protein continues to exert its actions even after internalization by the axons [53]. The *En*2 gene has also been implicated in early muscle development, with expression in the murine mandibular arch myoblasts in particular, which give rise to the masseter, temporalis, and lateral and medial pterygoid muscles involved in jaw closure [54]. Germline mutations in *En* are therefore likely to have profound effects on embryological development.

Expression of *En* genes in the human foetus has been demonstrated in all of the neuronal groups of the mid-gestational medulla and cerebellum, and is thought to be crucially involved in the development and anatomic organisation of these structures [55]. A murine model of homozygous mutation of the *En*1 and *En*2 genes results in disrupted formation of the mesencephalon and metencephalon, with cerebellar hypoplasia, which mimics the anatomical findings in two unrelated infants who were born at term but died in early infancy from lack of central respiratory drive. It is believed that these cases resulted from a mutation or deletion in the *En* gene [56].

The arcuate nucleus at the ventral surface of the medulla oblongata which aids in the control of central chemoreception, cardioventilatory activity and blood pressure [57], also expresses the *En*2 gene. Lavezzi et al. demonstrated high EN2 protein expression in the arcuate nucleus of the 17th to 22nd gestational week human foetus, with decreased expression up to the first days after birth [58]. They also studied 13 cases of sudden infant death, where

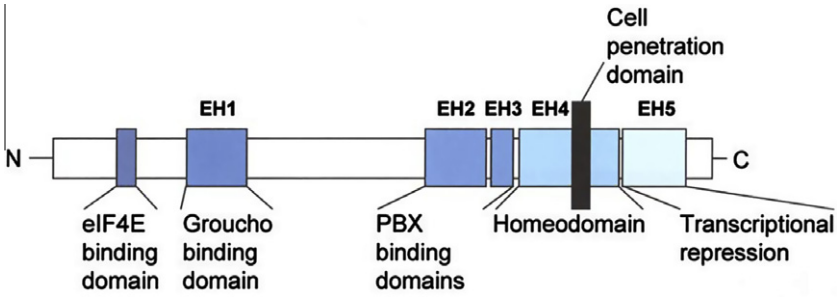

Fig. 1. Functional domains within the EN protein.

61% had hypoplasia of the arcuate nucleus with predominantly negative EN2 protein expression. Their findings support the role of *En*2 in normal human neuronal development and anatomic organisation, particularly of the arcuate nucleus.

The only known site of normal adult *En2* expression is in the nervous system, particularly the Purkinje neurones, where immunohistochemical staining identifies it in the nucleus [59,60]. Jankowski et al. showed that gene over-expression resulted in retardation in the maturation of the Purkinje cells, particularly in the timed development of their dendritic tree [61]. Owing to its expression in mesencephalic dopaminergic neurons, Rissling et al. investigated single-nucleotide polymorphisms (SNPs) in the promoter region or transcribed part of *En2*, in individuals with young-onset Parkinson's disease (YOPD) [62]. This suggested an association between SNP rs1345514 and the development of YOPD, although this has not yet been verified in an independent sample.

En2 has been linked to the neurodevelopmental disorder, autism, as it shares the chromosomal region 7q36.3 which is thought to be an autism susceptibility locus. There appears to be a genetic linkage in families with autistic members, but also broad similarities between the neuropathology seen in *En2* knockout mice and autistic individuals, including cerebellar hypoplasia, decreased Purkinje cells and an anterior shift in the position of the amygdala in the cerebral cortex [63–67]. Further cohort studies, mainly in Chinese and Indian populations, support this [68–70].

3. Engrailed in cancer

Over-expression of EN2 protein may be linked to tumour development in adult humans, particularly in breast, prostate, melanoma and ovarian cancers.

En2 has been identified as a potential oncogene in a breast cancer model. Martin et al. identified ectopic expression of *En*2 in a number of breast cancer cell lines but only 7.3% of human breast cancers [71]. There was no evidence of rearrangement or amplification of the gene, so the ectopic expression may result from epigenetic modification. Non-tumorogenic murine mammary cell lines were forced to express *En2*, and subsequently exhibited malignant characteristics, including a reduction in cell cycling time, a loss of cell to cell contact and a failure to differentiate in response to lactogenic hormones. The cell line induced mammary tumours when transplanted into the cleared mammary glands of syngeneic hosts. The same group showed that in a human breast cancer cell line, suppression of *En2* resulted in a significant decrease in their proliferation rate. They did not demonstrate expression of *En2* in normal breast epithelium, and *En1* was not demonstrated in cell lines or human breast tissue.

In the prostate, Bose et al. initially demonstrated *En2* over-expression in human prostate cancer cells as compared to normal prostate epithelial cells [72]. SiRNA-mediated down-regulation of *En2* in the cell lines resulted in a decrease in *PAX*-2 expression, and vice versa, and caused a dramatic decrease in prostate cancer cell proliferation, as with the previous findings in breast cancer cells [71]. Morgan et al. further identified *En2* in prostatic adenocarcinoma and demonstrated that it is not expressed in normal prostate tissue, normal tissue adjacent to the cancer, benign hypertrophy, or high grade prostatic intraepithelial neoplasia, a pre-malignant lesion [60]. Immunohistochemical staining of patient tumour biopsies showed that EN2 expression is most intense in the duct-like structures of tumours, the protein is present in the cytoplasm, sometimes in the basal membrane, but not in the nucleus. EN2 containing blebs were also identified in prostatic acini and ducts, confirming the known secretory properties of EN2. This group were able to identify EN2 in the urine of 66% of biopsy-proven prostate cancer patients, some of whom had undetectable levels of serum prostate specific antigen (PSA). This contrasted with <15% positivity in the low PSA control groups, prompting further research into its use as a potential biomarker, as discussed later. In a subsequent cohort of 125 Danish men with confirmed prostate cancer, 70% were positive for EN2 in their urine [73].

Preliminary work on *En2* expression in ovarian cancer has demonstrated high expression in some ovarian cancer cell lines, as well as in epithelial ovarian cancer tissue [McGrath, S.E. & Michael, A., unpublished work]. In a cohort of 118 epithelial ovarian cancer patients, 88.14% of tumours expressed EN2 whilst all normal ovarian tissue was negative. No correlation with tumour type, grade and stage was seen however EN2-expressing mucinous tumours showed improved overall survival ($P = 0.0253$). This data was supported by Immunohistochemical analysis of ovarian cancer tissue arrays which demonstrated positive EN2 in approximately 80% of ovarian cancer tissues, compared with low (<10%) or absent expression in normal tissues (Fig. 2). Analysis of the serum from 67 ovarian cancer patients and 42 age-matched female controls, showed that 20.9% of the former had antibodies against EN2 compared to 2.4% of the controls. Peptide specific cytotoxic T-lymphocytes were generated by a number of the identified EN2 epitopes, which may enable EN2 to be exploited as an immunotherapeutic target antigen.

Hypermethylation of the *engrailed* genes has been identified in several cancers although its specific role is yet to be characterised. Rauch et al. found that all four *HOX* gene clusters were preferential targets for DNA methylation in lung cancer cell lines, with additional hypermethylation of both *En1* and *En2* [74]. The exact significance of this is unknown however the authors hypothesised that such DNA methylation markers could be useful in early diagnosis of disease. Karpinski et al. determined the methylation status of three CpG islands at the 2q14.2 chromosomal band, including *En1*, in 148 sporadic colorectal cancers [75]. Generally 18% to 25% of sporadic colorectal cancers show CpG island methylator phenotype (CIMP), and the average number of methylated sites was significantly higher in these tumours, namely 70% *En1* methylation was seen in CIMP positive compared with 22% in CIMP negative tumours. The authors postulate that this hypermethylation, along with the other CpG islands, may contribute to the specific characteristics of CIMP positive tumours and their clinicopathologic features. Of note, these studies did not investigate subsequent *En* gene or protein expression. Hypermethylation of *En1* has subsequently been seen by Mayor et al. in 90% of colorectal tumours

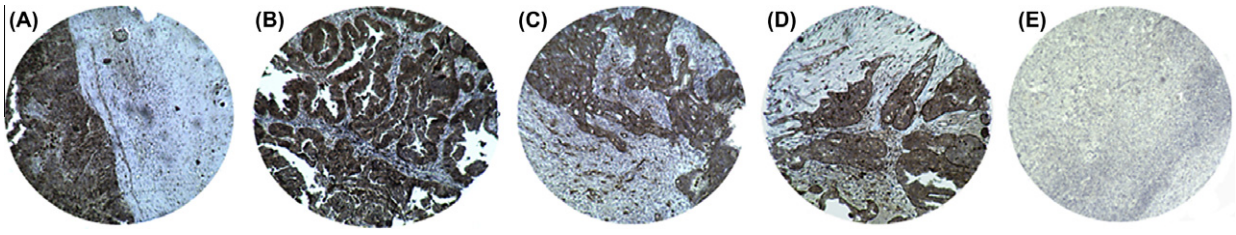

Fig. 2. EN2 antigen expression in ovarian cancer. Immunohistochemical analysis of EN2 expression (brown) on four examples of serous papillary adenocarcinoma (A–D) versus normal ovary (E).

[76], but also in astrocytoma [77] and prostate cancers [78]. *En2* hypermethylation, along with other homeobox genes, has been identified in the follicular lymphoma cell line, RL, and in ten primary follicular lymphomas [79]. However transcriptional down-regulation was not observed, indicating aberrant epigenetic regulation in follicular lymphoma. Although *En* hypermethylation is present in a number of malignancies its functional role is still unknown, as it does not appear to contribute to gene silencing. In fact Bell et al. demonstrated EN1 protein over-expression in cases of adenoid cystic carcinoma (ACC), where significant hypermethylation of the *En*1 gene was observed [80]. To date, this is the only published evidence of EN1 protein over-expression in malignancy. ACC represents a rare and progressive malignancy of the salivary glands in which the authors had previously observed significant hypermethylation at the transcriptional start site of the *En1* gene, prompting them to further evaluate EN1 protein expression [81]. In a cohort of 200 patients, they demonstrated positive EN1 expression in 58.8% of tubular, 56.5% of cribriform, and 85.7% of the solid histological subtypes. Nuclear staining of the inner ductal cells was observed with only faint expression in the cytoplasm of salivary ductal cells. The solid pattern was predominantly poorly differentiated and devoid of myoepithelial cells, suggesting that EN1 expression may correlate with a more aggressive tumour with poor prognosis. The group mentioned that they had also performed immunoreactivity on a triple-negative breast carcinoma microarray, which demonstrated similar results for high-grade basaloid breast carcinomas.

Table 1 summarises the current evidence linking the *Engrailed* genes and their protein products with cancer.

4. Engrailed as a potential biomarker

Several groups have reported the use of homeobox genes as diagnostic and prognostic biomarkers, as certain gene expression profiles can be linked to tissue specificity, association with early stages of carcinogenesis, and even therapy-resistant disease resulting in a worse prognosis. The majority of this work has been with the *HOX* gene family [82–85], however as more is known about *En*, its potential as a biomarker is growing.

There is currently a great deal of interest in the potential of EN2 as a biomarker in prostate cancer. Recent published evidence of its use as a tumour specific urinary biomarker for the early diagnosis of prostate cancer, showed that the presence of EN2 in urine was highly predictive of prostate cancer, with a sensitivity of 66% and a specificity of 88.2% [60]. The Prostate Cancer Prevention Trial recorded a sensitivity and specificity of 24% and 93% respectively for PSA, the current standard detection test for prostate cancer [86]. Hence it is clear that the use of PSA is limited, however it could be used in combination with urinary EN2 to reduce the need for prostatic biopsy.

Subsequently, Pandha et al. evaluated the relationship between levels of pre-treatment urinary EN2 and cancer volume in a Danish patient cohort who had undergone radical prostatectomy for prostate cancer. Seventy percent of patients were positive for EN2 in urine and there was a strong relationship between urinary EN2 and prostate cancer volume by linear regression ($P = 0.006$). Higher EN2 levels also correlated with advancing tumour stage ($P = 0.027$). Notably, no such relationships were observed with serum PSA however this may reflect the small cohort. It was hypothesised that EN2 may prove a useful diagnostic biomarker in prostate cancer, but also enable risk stratification due to the correlation with tumour volume [73].

Hypermethylation of *En1* and *En2* has been identified in several tumour sites, as previously discussed, and may have potential use as an early diagnostic marker. Mayor et al. were able to detect methylated *En1* in stool DNA from patients with colorectal carcinoma with 44% sensitivity and 97% specificity in patients with corresponding tumour methylation [76]. However the sensitivity fell to 27% when including the 27% of patients whose tumours did not show *En1* methylation. In serum, the sensitivity was only 11%. The authors concluded that the presence of a methylated *En1* CpG island in stool DNA could be developed as a diagnostic biomarker. *En1* was frequently methylated (65%) in primary prostate tumours, significantly differentiating cancer from normal tissue when combined with SCTR hypermethylation [78]. Combined methylation of these two genes may provide potential novel biomarkers for prostate cancer detection. Bell et al. initially suggested a potential role for *En1* methylation status as a biomarker in human salivary adenoid cystic carcinoma (ACC), given the correlation with histologic tumour grade, tumour location and patient outcome [81]. Subsequently they demonstrated a significant correlation between increased EN1 protein expression and a lower survival rate ($P = 0.014$) as well as a higher incidence of lymph node metastasis, suggesting its role as a prognostic biomarker [80].

Table 1
A summary of the evidence linking the *Engrailed* genes and antigens with cancer.

Evidence for involvement in cancer	Cancer type	Technique used	Reference
EN1 over-expression (*human tumours*)	Salivary gland (Adenoid cystic)	Immunohistochemistry	[80]
En2 promotes malignant characteristics (*murine mammary cell lines*)	Breast	Forced over-expression of *En2*	[71]
En2 expression required for cancer cell proliferation (*human cell lines*)	Breast	RNAi down-regulation of *En2*	[71]
	Prostate	RNAi down-regulation of *En2*	[72]
En2 over-expression (*human cell lines & tumours*)	Breast	Semiquantitative RT-PCR Immunohistochemistry	[71]
	Prostate	Semiquantitative RT-PCR Immunohistochemistry	[72,60]
	Ovary	Semiquantitative RT-PCR Immunohistochemistry	McGrath, S.E. & Michael, A. (unpublished)
EN2 secretion in urine (*human tumours*)	Prostate	ELISA	[60,73]
En1 hypermethylation (*human cell lines & tumours*)	Lung	Methylated-CpG island recovery	[74]
	Colorectal	Methylation-specific PCR	[75,76]
	Astrocytoma	Methylated-CpG island recovery	[77]
	Prostate	Methylated-CpG island amplification and microarray	[78]
	Salivary gland (Adenoid cystic)	Methylated CpG island amplification and microarray	[81]
En2 hypermethylation (*human cell lines & tumours*)	Lung	Methylated-CpG island recovery	[74]
	Follicular lymphoma	Methylation-specific PCR	[79]

5. Conclusion

The *Engrailed* sub-family of homeobox genes are essential in early embryonic development, but have also been identified in the normal adult nervous system, as well as in neurodevelopmental disease. However, the vast majority of *En1* or *En2* gene expression in the adult occurs in malignant tissue, particularly in breast, prostate and ovarian cancers. Methylation studies and identification of EN in urine have suggested a role for the gene and its protein product as potential biomarkers. It is hoped that further work may confirm associations between *En* expression and therapy-resistant, poor prognosis cancers, similar to that identified with other homeobox gene profiles.

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